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## Ultrastructure of Commissural Neurons of the Hilar Region in the Hippocampal Dentate Gyrus

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Previous studies have described the polymorph neurons in the hilus of the dentate gyrus at the light microscopic level and have indicated that many of those neurons are the cells of origin for both ipsilateral associational and commissural projections to the dentate gyrus. Because previous studies have not described the ultrastructural characteristics of the hilar neurons, we identified these features of the commissural neurons in the hilus. The method of retrograde transport of horseradish peroxidase (HRP) was utilized with a silver staining technique for HRP intensification. Two populations of labeled commissural neurons were observed in electron microscopic preparations of the contralateral hilus. One type consisted of cells with somata that exhibited round or oval nuclei with no intranuclear inclusions and formed symmetric axosomatic synapses. The main dendrites of those neurons were thick and tapering. In contrast, the other type of labeled neuronal soma had infolded nuclei containing intranuclear rods or sheets, displayed both symmetric and asymmetric axosomatic synapses, and had dendrites that were less thick and generally aspiny. In those same preparations, labeled commissural axon terminals formed synapses with dendrites and dendritic spines in the hilus and molecular layer and with somata in the granule cell layer. From the results of this study it appears that there are two distinct populations of commissural hilar neurons: one type resembles the morphology of the spiny CA3 pyramidal neuron, a type of excitatory projection cell, and the other type is similar to the dentate gyrus basket cell, a local circuit neuron associated with GABAergic inhibition. This latter cell type provides further support for the notion that some commissural neurons are inhibitory.

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Abbreviations: DAB—diaminobenzidine, GL—granule cell layer, HRP—horseradish peroxidase, WGA—wheat germ agglutinin.

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## INTRODUCTION

The polymorph neurons in the hilus of the dentate gyrus have been extensively described at the light microscopic level in the Golgi studies of Amaral (1) who showed approximately 20 cell types. Some characteristics of those cells include somata with a wide range of shapes and sizes, axons which arborize both in the hilus and granule cell layer, and dendrites either with spines (e.g., fusiform cells, oviform cells, large multipolar cells, and mossy cells) or without (e.g., spheroid cells, fusiform cells, stellate cells, small multipolar cells, and cells with ascending and descending axons). Horseradish peroxidase (HRP) labeling studies (3, 28, 29) and techniques using double labeling with fluorescent markers (27) indicated that at least 80% of those neurons are the cells of origin for ipsilateral associational and commissural projections to the dentate gyrus.

The ultrastructural characteristics of many types of neurons in the hippocampal formation have been described (4, 11, 14, 16, 17, 21, 23-25). The neurons that were analyzed in those studies included the granule and basket cells of the dentate gyrus and the pyramidal cells and interneurons of the hippocampus. The granule cells exhibit somata with oval or round nuclei that are rarely indented or infolded and contain no intranuclear inclusions except for heterochromatin and prominent, peripherally situated nucleoli. A thin rim of cytoplasm around the nucleus contains typical organelles except for Nissl bodies, the clustered cisternae of granular endoplasmic reticulum. The pyramidal cells resemble the granule cells in that they have round nuclei and no intranuclear rods or sheets. However, the somata of pyramidal cells are twice as large as the somata of granule cells and therefore have a more developed perikaryal cytoplasm. Spines are present on the dendrites of both pyramidal cells and granule cells and both cell types are projection neurons. In contrast, the features of the dentate gyrus basket cells, and other local circuit neurons in the hippocampus, differ from those of the above two hippocampal cell types in that the local circuit neurons generally have infolded nuclei, intranuclear rods or sheets, and aspiny dendrites.

Our purpose was to identify and characterize the commissural neurons of the hilus at the electron microscopic level because previous studies described only their light microscopic features. The use of HRP facilitated an analysis of the somata and dendrites of commissural cells to determine their ultrastructural features.

## MATERIALS AND METHODS

Thirty-three adult Sprague-Dawley albino rats from 250 to 350 g were used. After injections of 35% chloral hydrate (0.1 mg/100 g body weight, i.p.) the animals were injected with 0.10 to 0.20  $\mu$ l 30% HRP in distilled

water with a Hamilton syringe. The injections were made into the region of the dentate gyrus using stereotaxic guidance. The injections were made in 0.025- $\mu$ l increments and were completed in 10 to 20 min. The tip of the syringe then remained in place for 15 min before withdrawal from the brain. After a 48-h survival time, the rats were again anesthetized with chloral hydrate and fixed by intracardiac perfusion with 0.9% saline followed by a solution of 4% paraformaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer (pH 7.2). The perfused rats were refrigerated overnight at 4°C, and the brains removed from the cranium the following day.

Each brain was sectioned coronally on a vibratome at a thickness of 100  $\mu$ m. The sections that contained the hippocampus were processed using diaminobenzidine (DAB) and H<sub>2</sub>O<sub>2</sub> followed by two washes of phosphate buffer for 10 min each. Two methods were used for light microscopic analysis. One consisted simply of dehydrating sections in ethanol, clearing them in xylene, mounting them onto glass slides, and coverslipping. The other method involved a modified silver staining technique (10, 20) for HRP intensification. Briefly, the 100- $\mu$ m sections were washed in distilled H<sub>2</sub>O after DAB processing and refrigerated overnight in a solution of 5% HCl and 15% thioglycolic acid. The following day, the sections were washed in distilled H<sub>2</sub>O and placed in a physical developer (2.5% sodium carbonate, 0.1% silver nitrate, 0.095% ammonium nitrate, 0.5% tungstosilicic acid, and 0.013–0.125% Formalin). The intensity of staining was controlled by varying the time of development from 10 to 40 min. The reaction was stopped with incubation in 1% acetic acid followed by washes in distilled H<sub>2</sub>O. The sections were then placed in a 0.2% gold chloride solution, briefly washed, and incubated in 5% sodium thiosulfate, followed by another wash with distilled H<sub>2</sub>O. Finally, the sections were dehydrated in ethanol, cleared in xylene, mounted onto glass slides, and coverslipped.

For analysis in the electron microscope, blocks of tissue that contained HRP-positive cell bodies were cut from the DAB-processed unintensified and intensified sections and postfixed in 2% OsO<sub>4</sub>, dehydrated in ethanol, and embedded in Epon resin. Ultrathin sections obtained from these blocks were examined in the electron microscope. A total of eight brains yielding approximately 20 sections each were analyzed with the electron microscope and 45 HRP-labeled cells were examined.

## RESULTS

Injections of HRP that were placed into the right dorsal hippocampus displayed large deposits of reaction product in the CA3 region and hilus of the dentate gyrus in light microscopic preparations. The injection site itself was relatively large so that label would spread into the molecular layer, a major site of termination of commissural axons. Animals in which the HRP

injection site was outside the dentate gyrus were not processed for further analysis.

*Structures Labeled with Transported Horseradish Peroxidase.* Cells labeled with HRP were found in the contralateral CA3 region and in the hilus of the contralateral dentate gyrus. Because we were concerned with the morphology of the polymorph cells of the hilus, a description of the pyramidal cells of the CA3 region is not included in this report. The HRP-labeled cells were scattered throughout the hilar region but were frequently found beneath the granule cell layer (GL). These HRP-labeled commissural neurons were found to be projecting from the dentate gyrus in the dorsal hippocampus of one side to that of the other. However, using serial sections of the hippocampus, labeled cells could be found also in the contralateral ventral hippocampus.

The somata of HRP-labeled cells in the hilus displayed a wide variety of shapes including fusiform, triangular, and stellate (Figs. 1, 2). Their sizes varied, the diameters ranging from 10 to 30  $\mu\text{m}$ . Usually two to five labeled dendrites were stained for each labeled neuron and they were oriented in various directions. The somata subjacent to the GL had dendrites that were parallel to its long axis (Fig. 2). Typically the dendrites appeared to taper and sometimes branch while following a somewhat tortuous course. The longest observable dendrites extended to 55  $\mu\text{m}$  from their somata. Dendritic spines did not stain with this silver intensification technique. In addition, granule cells, basket cells, and local circuit neurons in the molecular layer were unlabeled in these preparations (Fig. 1).

Axons labeled with HRP were observed throughout the dentate gyrus on the side contralateral to the injection site (Fig. 1). These axons were limited to mainly the inner one-fourth of the molecular layer but extended into the inner one-third in some regions. They were oriented in many different directions with most coursing parallel to the GL. A single axon could be followed as far as 270  $\mu\text{m}$  along the length of the molecular layer-GL boundary. Labeled axons were also present in the GL where they appeared to be in continuity with axons in the molecular layer (Fig. 3). The angle of the branching of these axons suggests that they arose from axons in the molecular layer. In contrast, other GL axons appeared to display branching from hilar axons. Axons or branches of axons could be followed throughout the GL to lengths of 100  $\mu\text{m}$ . Some axons appeared to form terminal bulbs in the GL (Fig. 3). Inside the hilus, the commissural axons labeled with HRP were found throughout the hilar region but were concentrated beneath the GL. Generally, these axons had an appearance similar to axons in the molecular layer. However, the density and extent to which the axons could be followed in the hilus were less than that of the other labeled axons.

*Electron Microscopic Observations.* The heavy deposit of silver in the intensified sections did not facilitate identification of structures at the electron

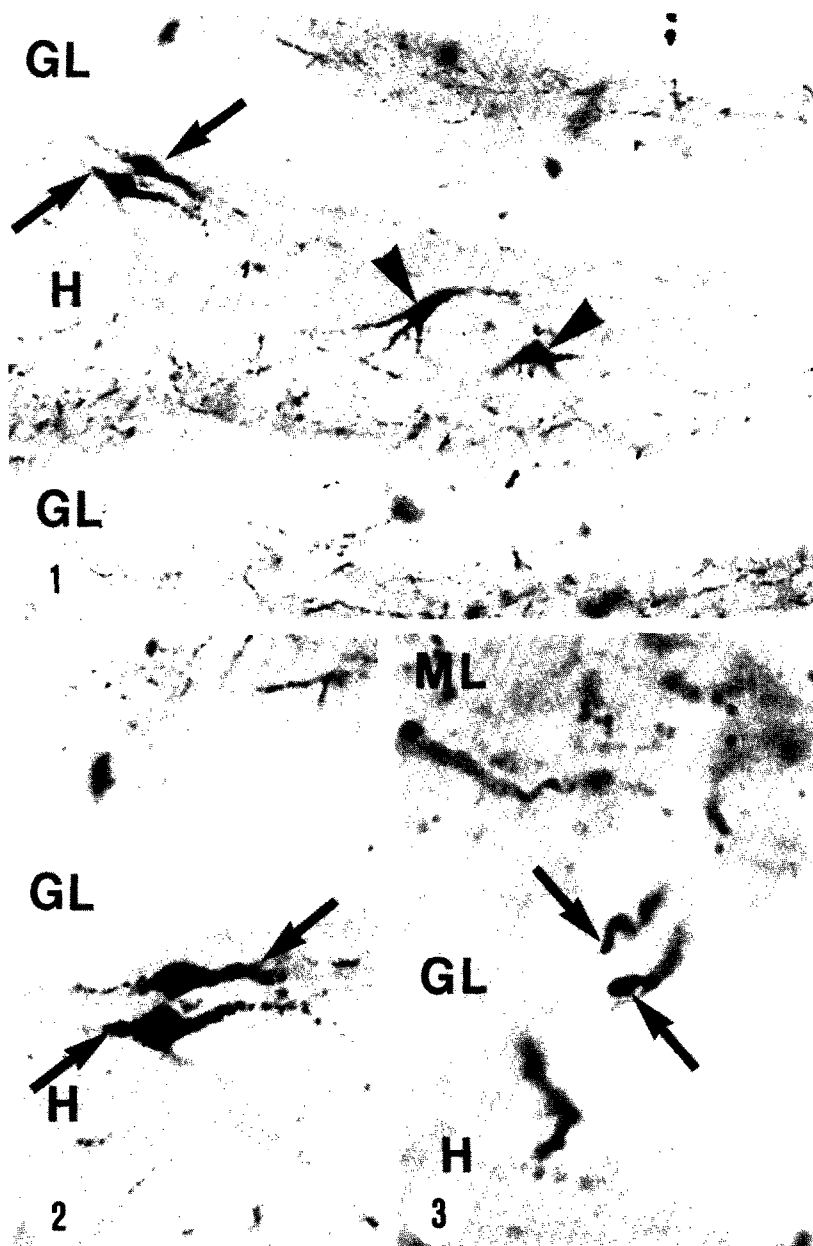


FIG. 1. Light micrograph of HRP-labeled neurons in the hilus of the dentate gyrus contralateral to the side of injection. Two bipolar fusiform-shape cells (arrows) were observed beneath the granule cell layer (GL) in the hilus (H). Also in the plane of focus were two multipolar neurons (arrowheads) in the center of the hilus. Labeled commissural axons were seen above, below, and occasionally within the unstained GL. Silver intensification method,  $\times 180$ .

microscopic level, although the HRP reaction product itself could be easily located. Therefore, for electron microscopic purposes, sections processed with the DAB method alone were used.

The HRP reaction product was identified in preparations by its homogeneous, electron-dense appearance in the electron microscope. It was localized to various irregularly shaped organelles about 75 to 605 nm in diameter including ovoid or round vesicles, dumbbell or cup-shaped organelles, and vacuolated cisternae of the agranular reticulum. In some cases, a membrane appeared to surround a labeled vesicle. The HRP-containing granules were readily distinguished from the typically electron-opaque lysosomes in non-labeled somata because the HRP-filled granules were more electron-dense and occurred in greater numbers. In addition, the diameters of the lysosomes were greater than the HRP-labeled granules. Although lipofuscin granules are also electron-dense, their fine laminar structure and electron-lucent areas distinguished them from the HRP-containing granules. Using these criteria for the identification of HRP labeling, two types of labeled neurons were found in the hilus.

These two types of labeled cells were intermixed throughout the hilus. One type consisted of cells with somata having features similar to those of the CA3 pyramidal neurons. These labeled somata with diameters of 10 to 30  $\mu\text{m}$  exhibited oval or round nuclei, no intranuclear rods or sheets, and cytoplasm rich in typical organelles (Fig. 4). The axon terminals that contacted these somata formed symmetric synapses (Figs. 5, 6). The other type of cell closely resembled the interneurons of Ammon's horn and the basket cells of the dentate gyrus. The somata of these labeled cells exhibited infolded nuclei, intranuclear rods or sheets, and a perikaryal cytoplasm rich in organelles including clusters of granular endoplasmic reticulum (Figs. 7, 8). This latter cell type was found throughout the hilus and displayed a variety of shapes. The horizontal hilar cell, located subjacent to the GL as well as deeper in the hilus, was a prime example of this cell type. Labeled horizontal hilar cells displayed extensive nuclear infoldings and prominent nucleoli. Moreover, the perikaryal cytoplasm of the horizontal hilar cells contained an abundance of mitochondria, scattered cisternae of the Golgi complex, and a large amount

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FIG. 2. Enlargement of two HRP-labeled commissural hilar neurons from Fig. 1 located subjacent to the granule cell layer (GL)-hilar (H) border. Granular reaction product extended into dendritic processes (arrows).  $\times 320$ .

FIG. 3. Light micrograph of HRP-labeled commissural axons. Numerous axons were present in the molecular layer (ML). Note the varicosities (arrows) on two of the labeled axons within the unstained granule cell layer (GL). Another axon in the GL appeared to arise from the hilus (H).  $\times 1280$ .

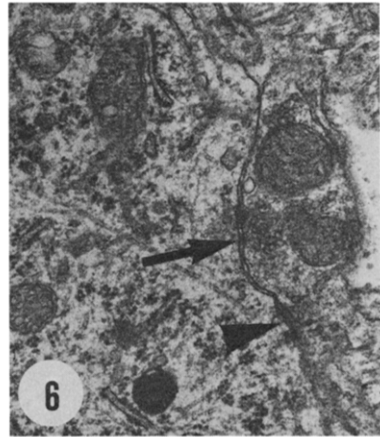
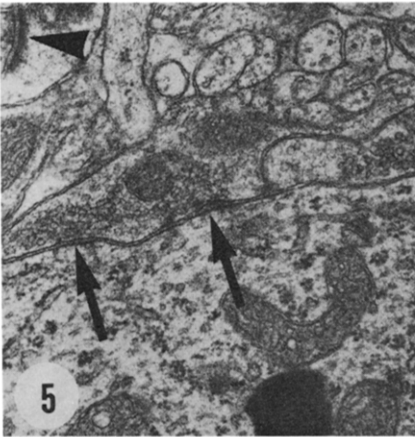
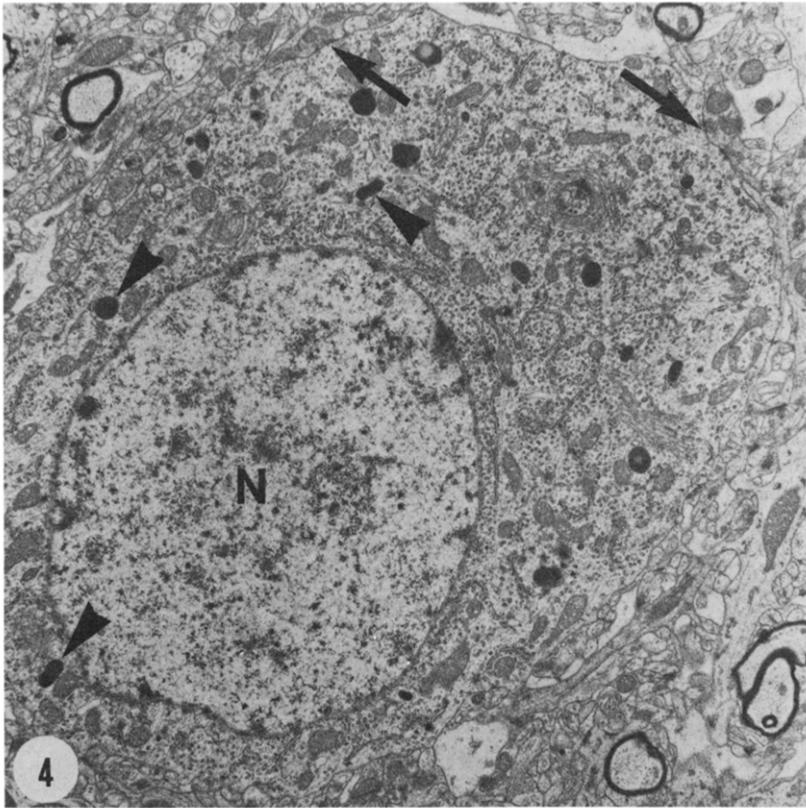


FIG. 4. Electron micrograph of an HRP-labeled soma of a commissural hilar cell. The nucleus (N) was round and contained no intranuclear rods or sheets. The reaction product for HRP was localized to membrane-bound vesicles (arrowheads). Numerous mitochondria, cisternae of the granular endoplasmic reticulum, and vesicles of the Golgi apparatus were contained within the perikaryal cytoplasm. Sites of synaptic contact (arrows) with this soma are shown at higher magnifications in Figs. 5 and 6.  $\times 9400$ .



of granular endoplasmic reticulum often grouped into Nissl bodies. In addition, the somata of horizontal hilar cells as well as the other somata of this second cell type were contacted by a mixture of terminals. Most of these terminals formed asymmetric axosomatic synapses whereas others formed symmetric synapses (Figs. 9, 10).

The main dendrites of these two types of neurons were often found to contain HRP-filled granules. The loci of these labeled granules, however, were usually limited to the proximal portions of the dendrite. The main dendrites that could be followed from the cell bodies of the first type of neuron were thick, tapering, and spiny. Those of the second type of neuron mentioned above were less thick and generally aspinous, although sessile spines were found on one labeled dendrite of this second cell type. In general, most dendrites were difficult to follow, even in serial sections, because they took tortuous routes. An obvious exception was the hilar horizontal cell that was located within an area 50  $\mu\text{m}$  beneath the GL.

Axon terminals labeled with HRP could be found in the hilus, GL, and molecular layer. The HRP-filled granules in the terminals were distinguished from dense core vesicles on the basis of electron opacity and size. The HRP granules exhibited greater electron density than dense core vesicles and were larger in size (100 to 200 nm diameter) than both synaptic vesicles (30 to 60 nm diameter) and dense core vesicles (60 to 100 nm). The HRP-labeled granules could also be followed in a number of serial sections whereas dense core vesicles could not. Labeled terminals were found to synapse with dendrites and dendritic spines in the hilus (Fig. 11) and molecular layer and with somata in the GL (Fig. 12).

## DISCUSSION

*Light Microscopy.* Our results confirm and extend the light microscopic findings of previous studies concerning the origin and termination of the commissural projection of the dentate gyrus. Briefly, the retrograde transport of HRP indicated that the polymorph cells of the hilar region are the origin for contralateral commissural projections to the dentate gyrus of the rat. These findings are in agreement with the observations of others using trans-

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FIG. 5. Enlargement of a terminal that formed symmetric synapses (arrows) with the soma of the commissural hilar cell in Fig. 4. In contrast, a neighboring terminal formed an asymmetric axodendritic synapse (arrowhead).  $\times 30,000$ .

FIG. 6. Electron micrograph of another symmetric axosomatic synapse (arrow) onto the soma of the cell in Fig. 4. This terminal appeared to have another active synaptic site (arrowhead), but the membranes were grazed obliquely at this latter point of contact.  $\times 30,000$ .

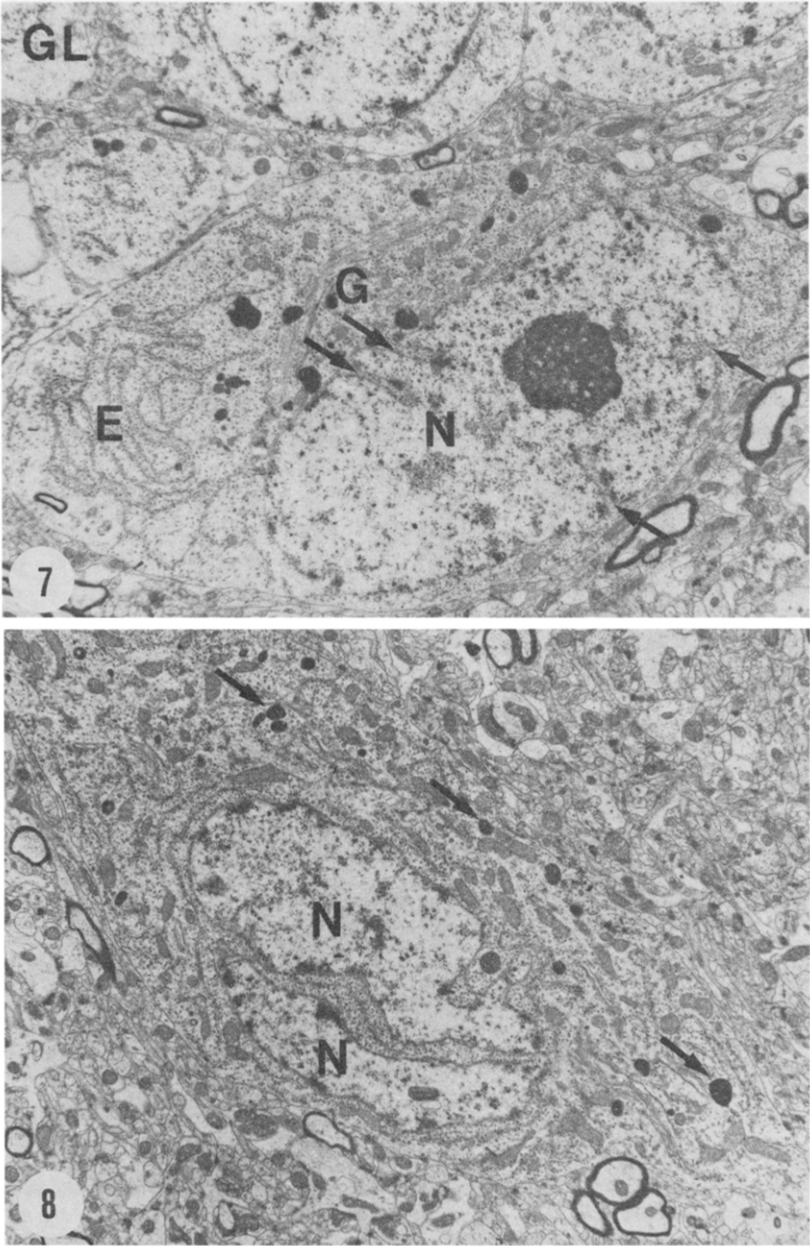


FIG. 7. Electron micrograph of an HRP-labeled commissural hilar horizontal soma located beneath the granule cell layer (GL). The nucleus (N) exhibited several infoldings (arrows) and displayed a prominent nucleolus. The perikaryal cytoplasm contained a large number of organelles including numerous clusters of granular endoplasmic reticulum (E), cisternae of the Golgi complex (G), and multishaped vesicles filled with HRP-positive reaction product.  $\times 8000$ .

ported HRP methods in the rabbit (3), mouse (29), and rat (28). Results with other techniques including fluorescent double labeling (27), autoradiography (12), and the Fink-Heimer silver impregnation method (14) also support those conclusions.

The results with anterograde transport of HRP showed that commissural axons terminated in the inner one-fourth of the molecular layer and in the hilar region. These data are consistent with results reported by others (3, 12, 14, 16, 18), although the density of terminals was reported to be much less inside the hilus in those other studies. A new finding in our study was the observation of axons branching into and coursing through the granule cell layer. These GL axons appeared to arise from commissural axons in both the molecular layer and hilus. Varicosities of these axons in the GL may represent synaptic terminals (see below) and indicate a direct commissural input to the somata of granule cells, an observation not previously reported. These findings could also be explained by a phenomenon whereby the HRP transported retrogradely from the contralateral side could have labeled hilar neuronal somata which then might transport the HRP via their ipsilateral associational pathway resulting in the appearance of labeled axons coursing through the GL. Although this possibility exists, it probably did not occur because no labeled axons could be observed to emanate from any labeled cells. In addition, none of the axons found inside the GL could be traced back to the HRP-labeled neurons in the hilus. In a recent study where trans-neuronal transport of HRP was reported in mammals, the HRP utilized was conjugated to the lectin, wheat germ agglutinin (WGA) (2). Thus, it seems plausible that the reported enhanced transport of WGA-HRP by neurons accounts for such a transneuronal conveyance. Because WGA-HRP was not used in the present study, it is likely that no transneuronal transport occurred. Therefore, the labeled axons in the GL are probably derived from commissural neurons in the contralateral hippocampal formation.

Another new finding involves the topography of the commissural projection. It is known that cells in the dentate gyrus in the dorsal hippocampus of one side project to that of the other side (28). In our study, labeled cells were found in the contralateral dentate gyrus of the ventral hippocampus in serial sections after dorsal hippocampal injections. These findings possibly indicate that the commissural projections have a more diverse origin than previously

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FIG. 8. Electron micrograph of another HRP-labeled commissural fusiform-shape hilar soma. This labeled soma was located deeper in the hilus than the one in Fig. 7. Because of the great amount of infolding in the plane of this section, the nucleus (N) appeared bilobed. The proximal portions of two main dendrites were observed extending from the soma at the upper left and lower right corners of this photomicrograph. Vesicles containing HRP-positive reaction product (arrows) were found throughout the soma.  $\times 6500$ .

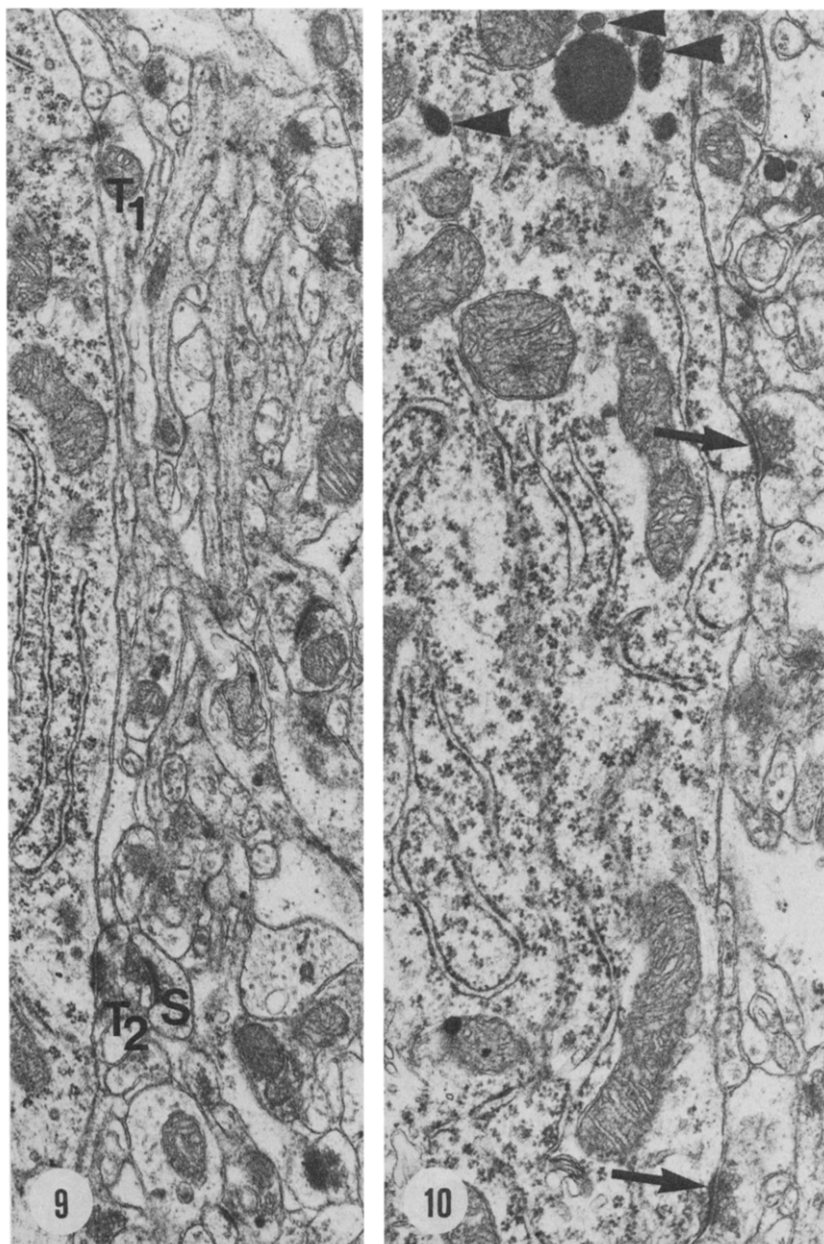


FIG. 9. Enlargement of two terminals that formed axosomatic synapses in the lower right hand corner of the cell in Fig. 8. One terminal ( $T_1$ ) formed a symmetric synapse and the other terminal ( $T_2$ ) formed an asymmetric synapse with the soma as well as with an adjacent spine (S).  $\times 25,500$ .

FIG. 10. Electron micrograph of two asymmetric synapses (arrows) that contacted the proximal dendrite of the commissural cell in Fig. 8. At this magnification the HRP reaction product was clearly observed within membrane-bound structures (arrowheads).  $\times 30,000$ .

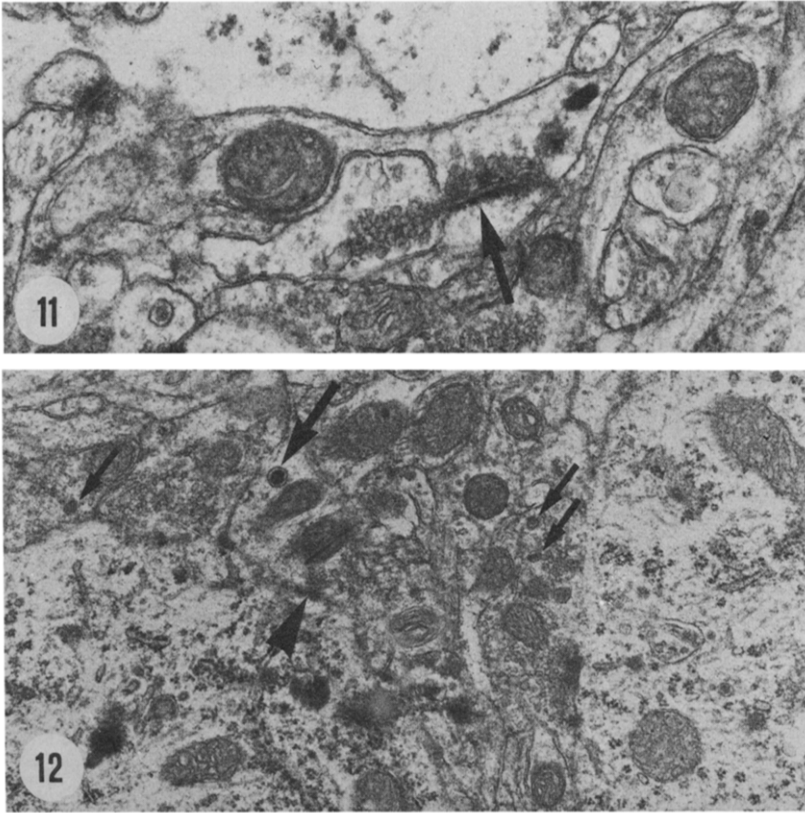


FIG. 11. Electron micrograph of an HRP-labeled commissural axon terminal in the hilus that formed an asymmetric synapse (arrow) with a dendritic spine.  $\times 38,000$ .

FIG. 12. Electron micrograph of an HRP-labeled commissural axon terminal apposed to the soma of a granule cell at the border with the hilus. An HRP-filled granule (large arrow) appeared within the terminal. This granule displayed a greater electron density and larger size than the typical dense core vesicles (small arrows) that were observed in other terminals. Although the synaptic cleft was not apparent in this section, the synaptic vesicles in the labeled terminal were accumulated adjacent to a probable active zone (arrowhead).  $\times 24,500$ .

thought. These data are different from neocortical commissural projections which project exclusively to homologous cortical areas (15).

*Electron Microscopy.* The main focus of this study involved the ultrastructure of commissural hilar neurons as determined by the examination of HRP-labeled somata and dendrites with the electron microscope. The identification of the HRP reaction product followed the criteria established by previous

investigators (8, 13, 19) as to appearance, localization, size, shape, and electron density of the HRP reaction product. Those criteria helped to distinguish HRP-filled granules from both lysosomes and lipofuscin granules.

The results of our study describe for the first time the ultrastructure of some hilar neurons in the dentate gyrus. On the basis of morphology, we observed two general types of HRP-labeled commissural cells scattered throughout the hilar region: one with somata that exhibit round or oval nuclei with no intranuclear inclusions and with symmetric axosomatic synapses, and the other type with somata that have infolded nuclei containing intranuclear sheets or rods and a mixture of asymmetric and symmetric axosomatic synapses. The first type of cell resembles the CA3 pyramidal neuron and to some extent the granule cell; the latter type resembles the morphology of the basket cells of the dentate gyrus. These latter cells are local circuit neurons which form synapses with granule cells in their proximal vicinity (23).

The spinous nature of the dendrites of labeled commissural neurons in the hilus was not always determined because only the very proximal portions of the dendrites were observable with the electron microscope due to their tortuous nature. From available data, however, the dendrites of the first type of neuron appear to be thick and spiny, again similar to those of CA3 pyramidal projection neurons. The second type of neuron exhibits aspiny dendrites, another characteristic shared by the local circuit neurons of the dentate gyrus.

Commissural axon terminals labeled with HRP were observed on dendrites in the molecular layer and hilus. These results are consistent with previous data from degeneration studies (5, 14, 18, 22). Labeled terminals were also observed to appose the somata of granule cells and this finding indicates a direct input from commissural hilar neurons.

Immunocytochemical results have shown that a substantial number of neurons in the hippocampus and dentate gyrus are GABAergic (24). More recent findings indicated that at least 60% of hilar neurons contain glutamate decarboxylase and thus probably use GABA as an inhibitory neurotransmitter (26). These data indicated that some GABAergic hilar neurons are projection cells. Taken together with the ultrastructural observations from the present study, it seems probable that there are two populations of commissural hilar neurons. One population is probably composed of inhibitory, GABAergic cells that resemble the morphology of local circuit neurons of the hippocampus and dentate gyrus but are actually projection neurons. The other population consists of cells that resemble CA3 pyramidal projection neurons in terms of both their structure and their excitatory commissural function (9). Recent electrophysiologic data support the notion that some inhibitory projection neurons exist in the commissural pathway (6, 7). Future studies will combine both retrograde tracers and immunocytochemistry techniques in the same

preparation to demonstrate the morphology of these presumed GABAergic, commissural projection neurons.

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